PCT
WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/00, 15/85, 15/82
A01K 67/00, C12N 9/64, 15/12
C12N 15/15, 15/89

A1

(11) International Publication Number: WO 92/11358

(43) International Publication Date: 9 July 1992 (09.07.92)

(21) International Application Number: PCT/GB91/02318

(22) International Filing Date: 24 December 1991 (24.12.91)

(30) Priority data:
9028062.9 24 December 1990 (24.12.90) GB

(71) Applicant *(for all designated States except US)*: THE AGRICULTURAL AND FOOD RESEARCH COUNCIL [GB/GB]; Wiltshire Court, Farnsby Street, Swindon SN1 5AT (GB).

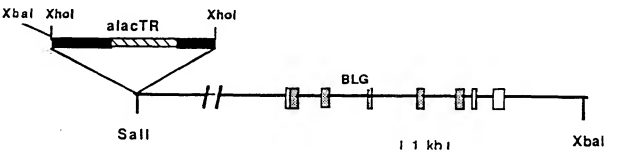
(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : CLARK, Anthony, John [GB/GB]; 29 Broomieknowe, Lasswade, Midlothian EH18 1LN (GB).

(74) Agents: SHEARD, Andrew, Gregory et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: INCREASED EXPRESSION BY A SECOND TRANSFERRED SEQUENCE IN TRANSGENIC ORGANISMS



(57) Abstract

Transgenic animals and plants capable of expressing a desired protein can be prepared by co-introducing into an egg or embryo cell of an animal, or into suitable recipient cells of a plant, a first sequence, which encodes the desired protein, and a second, more efficiently expressed, DNA sequence. Expression efficiency is thereby conferred on the first sequence, leading to improved yield or targeting, or both. Co-introduction may be achieved by co-injecting a mixture of the two DNA sequences into a fertilised egg, in the case of an animal. The invention can be used to enhance the efficiency of expression of desired proteins, such as those having pharmaceutical activity in the mammary gland of a female transgenic animal.

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INCREASED EXPRESSION BY A SECOND TRANSFERRED SEQUENCE IN TRANSGENIC ORGANISMS

This invention relates to the production of transgenic organisms, DNA preparations useful in such a method and transgenic animals and plants produced thereby.

Transgenic animals for a number of purposes have been developed and work on their further development continues. Among their uses, transgenic animals offer a powerful approach for the production of recombinant proteins. In particular, as is disclosed in WO-A-8800239, the expression of genes encoding a protein of interest can be targeted to the mammary gland of transgenic farm animals such as sheep, goats or cattle, and the protein product can be harvested from their milk.

The use of transgenic animals for protein production in milk necessitates foreign genes being introduced into the germ line in such a way that their expression can be directed to the mammary gland. Targeting of expression to other organs or tissues is important in transgenic animals prepared for other purposes. Introduction of foreign genes into an animal's germ line has been demonstrated in WO-A-8800239, which describes the production of transgenic sheep carrying genes designed to express human Factor IX and human α_1-antitrypsin in milk. In order to direct expression of these transgenes to the mammary gland, the approach taken was to take regulatory sequences from milk protein genes and fuse them to the protein coding sequences of the product of interest. In practice, this has involved the use of transgenic mice as a model system to assess the performance of the various hybrid gene constructs; the much shorter generation time of mice enables experiments to be undertaken within a realistic time-scale. For a genetic construct to be useful, it must express in the chosen model system at a reasonable frequency and at suitable levels.

A number of genomic milk protein genes which can be regarded as natural (that is to say the same configuration as DNA sequences found in the genome of animals from which the genes were isolated; such isolated gene may be termed genomic clones and will usually contain introns) have been shown to express relatively efficiently in transgenic mice. Reference is made in particular to Example 7 of WO-A-8800239, which relates to the expression of the gene coding ovine β-lactoglobulin in transgenic mice. Other examples include the systems of Vilotte *et al* (*Eur. J. Biochem.* **186** 43-48 (1989)) and Bayna *et al Nuc. Acids. Res.* **18** 2977-2985 (1990)). These genomic clones thus appear to contain all the essential regulatory sequences required for directing expression efficiently to the mammary gland.

A number of hybrid genes comprising milk protein gene regulatory elements fused to DNA sequences encoding proteins of interest have also been assessed in transgenic mice; in WO-A-8800239 the proteins of interest were human Factor IX and human α_1-antitrypsin. In many cases such as this, however, the level of expression of the hybrid genes is well below that which would be optimal for commercial purposes. A common feature of such relatively poorly expressing constructs is that they were constructed using contiguous cDNA sequences and therefore lacked their natural introns. This problem was addressed and overcome in WO-A-9005188, which disclosed the use of a construct comprising a genomic sequence encoding an exogenous protein and containing some, but not all, of its natural introns. Such a construct performed relatively efficiently.

In some circumstances, however, it may be wished to avoid including natural introns in a construct. First, the DNA sequences for many proteins of interest are available only as cDNA sequences; indeed, it may be much easier to prepare a cDNA sequence from a corresponding mRNA sequence transcribed in a given tissue in abundance in a target tissue than to isolate a genomic DNA. Secondly,

some genes are very large, even if the proteins that they encode are not correspondingly large; too great a size makes the inclusion of most or all of the introns difficult as a practical matter.

There is therefore a need to enhance the efficiency of expression of a construct which normally expresses poorly as a transgene. Such poorly expressing constructs include, but are not limited to, those comprising cDNA sequences. The present invention relates to such a method and thereby provides a solution to at least some of the problems described above. While the invention has particular application to the production of transgenic animals, its use in the production of transgenic plants is also contemplated to solve comparable problems in their production.

According to a first aspect of the present invention, there is provided a process for the preparation of a transgenic animal or plant capable of expressing a first DNA sequence, the process comprising co-introducing into a cell or group of cells from which an animal or plant may be derived the first DNA sequence and a second DNA sequence, wherein the second DNA sequence is, when so introduced without the first sequence, capable of being expressed as, or regulating the expression of, a transgene with greater specificity and/or a greater frequency of expression and/or at a higher level than that at which the first sequence, without the second sequence, is capable of being expressed as a transgene, and allowing a transgenic animal or plant to develop from the cell(s).

The most commonly used method of introducing DNA into an animal cell for the purpose of transgenesis is injection (or microinjection, as it is sometimes termed). This is the method of choice for the production of trangenic animals by means of the present invention. Usually a few hundred linear molecules of DNA is directly microinjected into a pro-nucleus (often the male pro-nucleus) of a fertilised one

cell egg; microinjected fertilised egg may then subsequently be transferred into the oviducts of pseudo-pregnant foster mothers and allowed to develop. The invention is however not limited to this method of introduction; any suitable method can be used. Egg or embryo cells may be used, as may embryonic stem (ES) cells.

Transgenic plants are usually currently prepared by different procedures. Preferably, DNA is transformed into plant cells using a disarmed Ti-plasmid vector and carried by *Agrobacterium* by procedures known in the art, for example as described in EP-A-0116718 and EP-A-0270822. Alternatively, the foreign DNA could be introduced directly into plant cells using an electrical discharge apparatus. This method is preferred where *Agrobacterium* is ineffective, for example where the recipient plant is monocotyledenous. Any other method that provides for the stable incorporation of the DNA within the nuclear DNA of any plant cell of any species would also be suitable. Such methods include those suitable for species of plant which are not currently capable of genetic transformation.

It appears that introduction of DNA in the ways decribed above leads to integration (at least in a functional if not a structural sense) within the host's DNA in such a manner that the transgene becomes hereditable. Co-introduction may therefore lead to co-integration. Co-integration may take place in such a way that the first and second DNA sequences segregate together (ie co-segregate) in subsequent generations; this would be consistent with closely similar physical sites of integration in the genome.

The first and second DNA sequences are in one embodiment of the invention co-introduced by introducing a mixture of them (for example by injection, in the case of animal methodology) into the recipient cell. However, it is not necessary for the two sequences to be introduced simultaneously; instead, sequential introduction may be found to be acceptable in practice. Co-introduction may also be achieved

by covalently or otherwise linking the first and second DNA sequences: the two sequences may be linked in a single DNA molecule. In this embodiment of the invention it is possible to tailor quite precisely the construct to be introduced; for example, a first sequence may be sandwiched between two second sequences, and/or the tandem nature of the sequences (ie whether they are head to head or head to tail) can be fixed.

A process of the first aspect of the invention can therefore be seen to involve the co-introduction of a relatively inefficient, but desired, transgene with a relatively efficiently expressing transgene; this may lead to co-integration at the same site in the chromosome or at sites near to one another.

The first DNA sequence may comprise cDNA or other DNA encoding the desired protein and, if the purpose of the transgene is expression, sufficient regulatory sequences (for example including a promoter) operatively linked to the protein-encoding DNA to direct the expression, for example in a target tissue or organ. In the case when expression is being targeted at the mammary gland of a mammal, the regulatory sequence may be derived from a milk protein, particularly a whey protein such as β-lactoglobulin, α-lactalbumin or whey acidic protein. The desired protein may be any protein (which term includes glycoprotein) sought to be and capable of being, produced in a transgenic. High value proteins, such as those having pharmaceutical activity, are particular candidates for use in the present invention. Example include, but are not limited to, insulin, plasminogen activators, α_1-antitrypsin, blood factors such as Factor VIII and IX, Protein C and erythropoietin. The regulatory sequences used in association with the protein-coding DNA may be 5' and/or internal and/or 3' regulatory sequences.

The second DNA sequence is capable of being expressed, as a transgene, when introduced without the first sequence, with greater specificity and/or a greater

frequency of expression and/or at a higher level (often a higher mean level) than that at which the first sequence alone is capable of being expressed. The second DNA sequence is therefore relatively efficiently expressed, compared to the first DNA sequence. A greater efficiency of expression may be achieved by a greater frequency of expressions. A greater frequency of expression means a higher proportion of animals or plants or lines that now express the first sequence; this is clearly of importance both practically and economically.

The second sequence may be derived from or constituted by a gene, preferably complete with its associated regulatory sequences, normally expressed in the target organ. For example, if the target organ for expression is the mammary gland, the second DNA sequence is preferably derived from a milk protein gene, again particularly a whey protein gene such as β-lactoglobulin or α-lactalbumin or a casein gene such αS_1-casein. The gene may be the same as that from which the regulatory sequences used in the first DNA sequence are derived. The second sequence may be an artificial construct or a normal gene.

Transgenes may be expressed in many organs of animals by means of this invention; the mammary gland is only one example. In plants, transgene expression may be specific for a particular tissue, if desired.

According to a second aspect of the invention, there is provided DNA useful for the preparation of a transgenic animal or plant expressing a first DNA sequence, the DNA comprising, on the same or separate molecules, the first sequence and a second DNA sequence, wherein the second DNA sequence is, when introduced as a transgene without the first sequence, capable of being expressed or regulating expression with greater specificity and/or with a greater frequency of expression and/or at a higher level than that at which the first sequence, when introduced as a transgene without the second sequence, is capable of being expressed.

The first and second DNA sequences may be embodied on the same DNA molecule, in which case the sequences may be regarded as covalently linked, with or without a linker sequence. Alternatively, the first and second DNA sequences may be provided on different DNA molecules. In this case, the DNA may take the form of a mixture of such molecules or a kit of separate preparations of the molecules.

The invention is not limited to the presence of a single first sequence and a single second sequence. A plurality of relatively inefficiently expressing first sequences may be potentiated by one or more relatively efficiently expressing second sequence, and a plurality of second sequences may potentiate one or more second sequences. It is preferred that the number of relatively efficiently expressing second sequences be present in excess, compared to the relatively inefficiently expressing first sequence. For example from 2 to 5 or 10 copies of the second sequence may be present per copy of the first sequence.

According to a third aspect of the invention, there is provided a transgenic animal or plant capable of expressing a first sequence as a transgene, the said animal or plant having the first sequence and a second sequence, wherein the second sequence is such that, when introduced as a transgene without the first sequence, it is capable of being expressed or regulating expression with greater specificity and/or with a greater frequency of expression and/or at a higher level than that at which the first sequence, when introduced as a transgene without the second sequence, is capable of being expressed.

In the case of animals, suitable species include those which are (a) practicable to work with and (b), if the object is to use the invention for production of a desired molecule, are capable of giving sufficient yields for practical purposes. Small animals such as rodents including mice, rats, hamsters and guinea pigs may be

suitable for some applications; livestock animals such as cattle, sheep, goats and pigs may be preferred for others.

In the case of plants, the invention is applicable to a wide variety of species, both monocots and dicots, depending on the intended application.

Preferred features of the second and third aspects of the invention are as for the first aspect, *mutatis mutandis*.

The invention will now be illustrated by the following examples and contrasted with the comparative examples. The examples and comparative examples refer to the accompanying drawings, in which:

FIGURE 1 shows a restriction map of clone lambda SS1, which contains the gene for ovine β-lactoglobulin (BLG);

FIGURE 2 shows an SDS PAGE analysis of murine and ovine whey proteins (Comparative Example 2);

FIGURE 3 shows a Western blot of the gel of Figure 2 using rabbit anti-β-lactoglobulin serum;

FIGURES 4a and 4b show the construction of plasmid pBJ16 (Comparative Example 4);

FIGURE 5 shows Southern blots from mice resulting from co-injection of BLG and AATD and indicates the co-segregation of the two transgenes (Example 1); Band 1 is a BLG-specific band; and Band 2 is an AATD-specific band;

FIGURE 6 shows a Northern blot showing the result of hybridisation experiments indicating the tissue specific expression of an AATD transgene in mice according to the invention (Example 1);

FIGURE 7 shows a Northern blot showing the result of hybridisation experiments which reveal the presence of two transgenes in mice according to the invention; Band 1 is an AATD-specific transcript (~1600 nt); Band 2 is an AATB-specific transcript (~1400 nt); and Band 3 is a BLG-specific transcript (~800 nt);

FIGURE 8 is a Southern blot showing the co-segregation of FIXD and BLG (Example 2); Band 1 is a BLG-specific band; Band 2 is a FIXD-specific band; and Band 3 is a non-specific junction band;

FIGURE 9 shows the tissue-specific expression of FIXD in BIX lines (Example 2);

FIGURE 10 is a Northern blot illustrating detection of FIXD and BLG transcripts in BIX mice; Band 1 is an endogenous mouse fIX transcript (~2600 nt); Band 2 is a BLG-FIX transcript in FIXA51; Band 3 is a FIXD transcript; and Band 4 is a BLG transcript; and

FIGURE 11 shows the construction of a β-lactoglobulin/bovine α-lactalbumin construct (Example 3).

COMPARATIVE EXAMPLE 1

Where not specifically detailed, recombinant DNA and molecular biological procedures were after Maniatis *et al* ("Molecular Cloning" Cold Spring Harbor (1982)) "Recombinant DNA" *Methods in Enzymology* Volume **68**, (edited by R. Wu), Academic Press (1979); "Recombinant DNA part B" *Methods in Enzymology* Volume **100**, (Wu, Grossman and Moldgave, Eds), Academic Press (1983); "Recombinant DNA part C" *Methods in Enzymology* Volume **101**, (Wu, Grossman and Moldgave, Eds), Academic Press (1983); and "Guide to Molecular Cloning Techniques", *Methods in Enzymology* Volume **152** (edited by S.L. Berger & A.R. Kimmel), Academic Press (1987). Unless specifically stated, all chemicals were purchased from BDH Chemicals Ltd, Poole, Dorset, England or the Sigma Chemical Company, Poole, Dorset, England. Generally, all DNA modifying enzymes and restriction endonucleases were purchased from BCL, Boehringer Mannheim House, Bell Lane, Lewes, East Sussex BN7 1LG, UK.

[Abbreviations: bp = base pairs; kb = kilobase pairs, AAT = human α_1-antitrypsin; BLG = sheep β-lactoglobulin; FIX = factor IX; *E. coli* = *Escherichia coli*; dNTPs = deoxyribonucleotide triphosphates; restriction endonucleases are abbreviated thus e.g. *Bam*HI; the addition of -O after a site for a restriction endonuclease e.g. *Pvu*II-O indicates that the recognition site has been destroyed]

Preparation of Sheep Spleen DNA

Spleen tissue was procured from a freshly slaughtered Blackface/Suffolk lamb and nuclei were isolated essentially as described by Burch and Weintraub *Cell* **33** 65 (1983). Nuclear pellets were lysed in 0.3M NaCl, 10mM Tris.HCl, 10mM EDTA, 1% SDS pH 7.4 and 400 μg/ml Proteinase K (Sigma Co, Fancy Road,

Poole, Dorset BH17 7NH) and incubated for two hours at 37°C. Repeated phenol/chloroform extractions were performed until the preparation was completely deproteinised. The DNA was ethanol precipitated and spooled out using a glass rod, washed with 70% EtOH/30% TE (TE = 10mM Tris.HCl, 1mM EDTA pH 8.0), dried in air and resuspended in TE to a concentration of 1 mg/ml.

Construction of Sheep Spleen DNA Lambda Fusion Genes

The lambda phage EMBL3 (Frischauf *et al J. Mol. Biol.* 170 827 (1983)) was employed to construct the genomic library. 30 μg of bacteriophage DNA were digested with 5-fold excesses of the restriction enzymes *Eco*RI and *Bam*HI (supplied by Amersham International plc, Lincoln Place, Green End, Aylesbury, Buckinghamshire, England) using the conditions recommended by the manufacturer. After digestion, spermine hydrochloride was added to a concentration of 5mM to precipitate the lambda DNA. After incubation for one hour on ice the DNA was pelleted at 10,000g for 15 minutes in a bench microfuge, washed in 70% EtOH, 300mM NaAc, 100mM $MgCl_2$, repelleted and finally resuspended in TE at a concentration of 1 mg/ml.

Sheep DNA was partially digested with the restriction enzyme *Sau*3A (Amersham). 100 μg aliquots of the sheep DNA were digested with varying amounts of *Sau*3A [from 5-40 units] for 20 minutes at 37°C. The reactions were stopped by the addition of EDTA to 15mM. The degree of digestion was assessed by electrophoresis on 0.6% agarose gels. Suitably digested samples were pooled and loaded onto 38.0ml 10-40% sucrose gradients made up in 1M NaCl, 20mM Tris.HCl, 5mM EDTA at pH 8.0. These gradients were centrifuged in a BECKMANN SW 28 rotor at 26,000 rpm for 24 hours. (The expression BECKMANN SW 28 is a trade mark.) The sucrose gradients were fractionated from the top and 1ml fractions collected. The size distribution of DNA molecules

in each fraction was assessed by agarose gel electrophoresis, and fractions containing DNA molecules from 14-21 kb in size pooled. After a two-fold dilution in TE 2 volumes of EtOH were added and the DNA precipitated overnight at -20°C. The DNA was subsequently resuspended in TE to a concentration of 300 μg/ml.

7.5 μg of *Bam*HI/*Eco*RI cut EMBL3 and 2.5 μg of sheep spleen DNA which had been partially digested with *Sau*3A were mixed together in 50 μl of a solution containing 60mM Tris.HCl, 6mM $MgCl_2$, 10mM DTT, 0.01% gelatin, 0.25mM rATP and 25 units of T_4 DNA ligase (Boehringer Company, Boehringer Mannheim House, Bell Lane, Lewes, East Sussex) and incubated overnight at 14°C.

After ligation 1 μg aliquots of the DNA were packaged *in vitro* using a kit purchased from Amersham following the recommended procedure of the manufacturer. The packaged library was titred on *E. coli* strain ED 8654. The estimated size of the library was 5.7 x 10^6 plaque forming units (pfu). Immediately after titration, aliquots of the unamplified library were plated onto 10 x 22cm^2 petri dishes (megaplates) using *E. coli* strain ED 8654 at a density of approximately 50,000 pfu/plate.

Screening the Lambda Genomic Library

Plaque-lifts from the megaplates were performed according to the method of Benton and Davis (*Science* **196** 180 (1977)) onto 20cm^2 nitrocellulose membranes (Schleicher and Schull, Postfach 4, D-3354, Germany). A β-lactoglobulin cDNA clone (p931 - gift of J.C. Mercier, INRA, Jouey-en-Josas, Paris) was nick translated with ^{32}P dCTP to a specific activity > 10^8 dpm/mcg, by the method described by Rigby *et al (J. Mol. Biol.* **113** 237 (1977)). β-lactoglobulin cDNA

may be cloned as described by Mercier *et al* in *Biochimie* 67 959-971 (1985). The sequence of the p931 clone is given by Gaye *et al* in *Biochimie* 68, 1097-1107 (1986). Filters were prehybridised, hybridised and washed according to the method of Maniatis *et al* in *Cell* 15 687 (1978). The final wash was in 1 x SET at 68°C (SET is 0.15M NaCl, 2mM EDTA, 0.03m Tris.HCl pH 8.0). Filters were blotted dry and spotted with ^{32}P to orientate them before exposure to X-ray film. Regions containing positively hybridising plaques were positioned on the megaplates by reference to the ^{32}P spots picked using the sterile blunt end of a Pasteur pipette. The initial plaque lifts were titred on *E. coli* ED 8654 and plated onto 15cm diameter Petri dishes at a plaque density of approximately 500/plate. These plates were rescreened by the procedures described above and individual positively hybridising plaques were picked using a toothpick into 1.0ml of phage-buffer (phage buffer is 10mM Tris.HCl, 10mM $MgCl_2$, 0.01% gelatin, pH 7.4).

Preparation of Cloned β-Lactoglobulin DNA

0.4ml of the resuspended phage solution was added to *E. coli* ED 8654 (Borg *et al Mol. Gen. Genetics* **146** 199-207 (1976)) and plated out on 9cm diameter Petri dishes to obtain confluent lysis of the bacterial lawn. Confluent plates were obtained from which the top plating agar was scraped off into 10ml of phage buffer and incubated overnight with a few drops of chloroform. The bacterial debris was pelleted by centrifugation at 5000 rpm for five minutes and the phage stocks stored at 4°C. The stocks were titrated on *E. coli* ED 8654 to determine the pfu/ml figure.

8×10^7 pfu were absorbed onto 7×10^9 *E. coli* cells in 10ml of 10mM $MgSO_4$ at 37°C. After 15 minutes, 2.5ml aliquots were added to 100ml L Broth/10mM $MgSO_4$ in a one litre flask. The bacterial suspension was shaken vigorously for several hours and the OD_{540} was monitored every hour. Lysis, as determined by

a fall in the OD_{540}, occurred after several hours. When complete, 0.2ml chloroform was added to each 100ml culture and the culture left at 4 C overnight.

The bacterial debris was removed by centrifugation at 10,000 rpm for 15 minutes. 10 μg/ml RNAse A and 10 μg/ml DNAse I were added to the supernatant which was then incubated at 37°C for one hour. After this incubation NaCl was added to 40g/litre and polyethylene glycol (PEG) to 10%. The preparation was cooled to 4°C and left for at least two hours to precipitate the phage. The phage pellet was pelleted at 10,000 rpm for 15 minutes and resuspended in 16.0ml of phage buffer. 8.0ml of this suspension was layered upon a step gradient comprising 1.5ml 56% CsCl, 1.5ml 45% CsCl and 2.5ml 31% CsCl (dissolved in phage buffer) in a 14.0ml ultracentrifuge tube. The step gradients were centrifuged at 35,000 rpm for 1.5 hours in a swing-out rotor at 20°C. The phage band was removed with a needle and syringe and, to complete the purification of the phage particles, a second step gradient centrifugation was performed.

The purified phage particles were dialysed into 0.1M NaCl, 10mM Tris.HCl, 1mM EDTA pH 8.0 and then deproteinised by successive extractions with phenol and chloroform. NaCl was added to a final concentration of 0.3M and then the phage DNA precipitated by the addition of 2 volumes of EtOH. The DNA was pelleted by centrifugation at 10,000 rpm for 20 minutes, washed with 70% EtOH, 30% TE, dried and then resuspended in TE to a final concentration of 200-400 μg/ml.

Characterisation of Recombinant Beta-Lactoglobulin Clones

0.5 μg aliquots of the DNA preparations described above were restricted with a variety of restriction enzymes and the products of the single and double digests analysed by electrophoresis on 0.6% and 1% agarose gels. DNA on these gels

was transferred to nitrocellulose filters on to HYBOND membranes (Amersham International, Little Chalfont, Bucks) by the method of Southern (*J. Mol. Biol.* **98** 503 (1975)) and hybridised to ^{32}P labelled p931. (The word HYBOND is a trademark.) The procedure used was essentially as described above and the hybridised filters were analysed by autoradiography. Using a variety of restriction enzymes and specific probes from the 5' and 3' ends of p931 a restriction map was constructed in which the size and orientation of the β-lactoglobulin gene(s) was determined, (see Figure 1).

The identity of the β-lactoglobulin clones and the precise position of the 5' and 3' ends of the gene were directly confirmed by DNA sequencing. Using suitable restriction sites, fragments were subcloned into plasmid vectors and into M13 vectors. Sequencing was carried out using the dideoxy method of Sanger et al. (*PNAS* 74 5463 (1977)).

COMPARATIVE EXAMPLE 2

Expression of the Gene Encoding Ovine Beta Lactoglobulin in Transgenic Mice

Transgenic mice were generated essentially by the techniques described in Gordon and Ruddle, in Methods in Enzymology Vol 101 (1983), (Eds. Wu, Grossman and Moldave), Academic Press pp411-432. Several transgenic mice carrying the 16.2 kb *SalI* fragment of the clone lambda SS-1 (Fig. 1) were produced. One of these, B-Lac 7, a female was shown to carry 15-20 copies of the *SalI* fragment. B-lac 7 was mated a number of times and produced a number of offspring which inherited the SS-I sequences.

Milk was obtained from mice 8-12 days after the birth of a litter. This was

accomplished by intra-peritoneal injection of 0.3IU oxytocin (Sigma) & 7 μl/g animal of Hypnorm/Hypnovel (Fleckneil, *Vet. Rec.* Dec 10 1983, p574), after having previously removed the pups for a four hour period, waiting 20 minutes and then massaging the individual mammary gland by hand. Milk was collected in a 50 μl capillary tube.

The mouse milk was diluted 1:5 in distilled water, centrifuged briefly in a bench centrifuge to defat and the caseins precipitated by addition of 1N HCl to a final pH of 4.6. After centrifugation in a bench centrifuge the whey proteins were removed, precipitated with 5% trichloracetic acid and analysed by polyacrylamide gel electrophoresis according to Laemmli (*Nature* 277, 680-684 (1970). (Figure 2 shows an SDS PAGE analysis of murine and ovine whey proteins.) Lane 1, marker proteins; 2, normal mouse whey; 3, sheep whey; 4, normal mouse whey; 5, B-lac7 whey; 6, B-lac7 whey (2.5 x 5). The band corresponding to β-lactoglobulin in the marker track and in ovine whey is arrowed.) Anti-sera raised in rabbits against ovine β-lactoglobulin was used to detect ovine β-lactoglobulin by Western blotting (Burnett, *Anal. Biochem.*, 112, 195-203, (1981)) on samples resolved by gel electrophoresis. Figure 3 shows a Western Blot Analysis. The Western blot was reacted with rabbit anti-β-lactoglobulin serum and anti-rabbit Ig peroxidase serum. (Lane 1, marker proteins; 2, sheep whey; 3, B-lac7 whey; 4, normal mouse whey; 5, purified β-lactoglobulin; 6, Coomassie stained sheep whey (run n parallel)).

This analysis showed that large amounts of β-lactoglobulin were secreted into mouse milk, indicating that SS-1 was being expressed at high levels in B-lac 7. This clone presumably contains all the necessary sequences to ensure high levels of expression in the mammary gland of a transgenic mouse and can thus be expected to function as efficiently, if not more so, in the homologous species ie in a transgenic sheep. Consequently, fusion genes derived from this clone can also

be expected to express (efficiently) in the ovine mammary gland.

COMPARATIVE EXAMPLE 3

The procedure of Comparative Example 2 was followed, except that a 10.5 kb *SalI-XbaI* fragment from lambda SS-1 (Fig.7) was used in place of the 16.2 kb *SalI* fragment (see Simons *et al* (*Nature* **328** 530-532 (1987)). The 10.5 kb fragment derived from lambda SS-1 was cloned into plasmid pPoly1 (see WO-A-8800239 and Lathe *et al* (*Gene* **57** 193-201 (1987)) at the *XbaI* and *SalI* sites on that plasmid. The resulting plasmid was termed pSS1tgXS. High levels of expression were again obtained.

COMPARATIVE EXAMPLE 4

Elaboration of Beta-Lactoglobulin Fusion Genes

The strategy used for elaborating fusion genes comprising DNA sequence elements from the ovine β-lactoglobulin and DNA coding for α_1-antitrypsin to be expressed in the mammary gland is described in this example. Figures 4a and 4b summarise the procedure. The approach utilises sequences derived from a lambda clone, lambdaSS-1, which contains the gene for ovine β-lactoglobulin, and whose isolation and characterisation is outlined in WO-A-8800239 (Pharmaceutical Proteins Ltd) and by Ali & Clark (1988) *Journal of Molecular Biology* 199, 415-426.

Specifically, the elaboration of the construct AATD is described. This construct contains the cDNA for human α_1-antitrypsin flanked by BLG sequences. The 5' flanking sequences include the *SalI* to *PvuII*-0 BLG sequences. The fusion point between the BLG and AAT sequences is in the 5'-untranslated region of the BLG

first exon. The 3' flanking sequences comprise exons 6 and 7 of BLG and the 3' flanking sequences of the BLG gene as far as the *Xba*I site. This construct contains no introns and was designed to examine whether the 5' and 3' BLG sequences described above are sufficient to direct efficient mammary specific expression of cDNAs encoding human plasma proteins as exemplified by that for AAT.

Plasmid pSS1tgSpX

The gel purified *Sph*I - *Xba*I restriction fragment of about 6.6 kb from pSS1tgXS (described WO-A-8800239) was ligated using T4 DNA ligase to gel purified pPolyI (Lathe, Vilotte & Clark, 1987, *Gene* 57, 193-201) (also described in WO-A-8800239) digested with *Sph*I and *Xba*I. [The vector pPolyI is freely available from Professor R. Lathe, LGME-CNRS and U184 INSERM, 11 rue Humann, 67085, Strasbourg, France.] After transformation of competent *E. coli* strain DHRα (Gibco-BRL) the correct clone was identified by restriction enzyme analysis.

Plasmid pBJ5

The gel purified *Pvu*II restriction fragment containing the origin of replication from pSS1tgSpX was self-ligated using T4 DNA ligase in the presence of 1mM hexamine cobalt chloride, 25mM KCI [to encourage self-ligation (Rusche & Howard-Flanders (1985) *Nucleic Acids Research* 13, 1997-2008)]. After transformation of competent *E. coli* strain DHRα (Gibco-BRL) the correct clone was identified by restriction enzyme analysis.

Plasmid pUCβlacA

Two complimentary 44-mer oligonucleotides, synthesised by the Oswell DNA Service, Department of Chemistry, University of Edinburgh, and containing bases 117-159 of the ovine β-lactoglobulin cDNA sequence (Gaye *et al*, (1986)

Biochimie **68**, 1097-1107) were annealed to generate *SalI* and *StyI* complimentary termini. The annealed oligonucleotides were then ligated using T4 DNA ligase to equimolar amounts of a gel purified 457 bp *StyI* - *SmaI* fragment from β-Lg 931 (Gaye *et al*, op cit) and gel purified pUC19 (Pharmacia-LKB Biotechnology, Pharmacia House, Midsummer Boulevard, Central Milton Keynes, Bucks, MK9 3HP, UK) which had been digested with *SalI* - *SmaI*. After transformation of competent *E. coli* strain JM83 (see Messing (1979) Recombinant DNA Technical Bulletin, NIH Publication No. 79-99, 2, No. 2 (1979), 43-48) the correct recombinant was determined by restriction analysis.

Plasmid pBJ7

The gel purified HincII - *SmaI* restriction fragment from pUCβlacA was ligated using T4 DNA ligase to gel purified pBJ5 linearised by partial digestion with *SmaI*. After transformation of competent *E. coli* strain DH5α (Gibco-BRL) the correct clone was identified by restriction enzyme analysis.

Plasmid pBJ8

The gel purified *PvuII* restriction fragment containing the origin of replication from pBJ7 was self-ligated using T4 DNA ligase in the presence of 1mM hexamine cobalt chloride, 25mM KCI (to encourage self-ligation [Rusche & Howard-Flanders (1985) *Nucleic Acids Research* **13**, 1997-2008)]. After transformation into competent *E. coli* strain DH5α (Gibco-BRL) the correct clone was identified by restriction enzyme analysis.

Plasmid pUC8α1AT.73

The plasmid p8α1ppg containing a full length cDNA encoding an M variant of α_1-antitrypsin was procured from Professor Riccardo Cortese, European Molecular Biology Laboratory, Meyerhofstrasse 1, D-6900 Heidelberg, Federal Republic of Germany (Ciliberto, Dente & Cortese (1985) *Cell* **41**, 531-540). The strategy used

in the construct BLG-AAT or pSS1tgXSTARG, now known as AATA, described in WO-A-8800239 required that the polyadenylation signal sequence at the 3' end of the α_1-antitrypsin cDNA be removed.

The polyadenylation signal was removed in the following manner. Plasmid p8α1ppg DNA was digested with *PstI* and the digestion products were separated by electrophoresis in a preparative 1% agarose gel containing 0.5 μg/ml ethidium bromide (Sigma). The relevant fragment of about 1400 bp was located by illumination with a UV lamp (Ultra-Violet Products, Inc, San Gabriel, California, USA). A piece of dialysis membrane was inserted in front of the band and the DNA fragment subsequently electrophoresed onto the membrane. The DNA was eluted from the dialysis membrane and isolated by use of an 'ElutipD' [Scleicher and Schull, Postfach 4, D-3354, Dassel, W. Germany], employing the procedure recommended by the manufacturer. The gel purified 1400 bp *PstI* fragment was digested with the *TaqI*, electrophoresed on a preparative 1% agarose gel as described above. The *TaqI* - *PstI* fragment of approximately 300 bp comprising the 3' end of the α_1-antitrypsin cDNA including the polyadenylation signal sequence was eluted and purified using an Elutip as described above, as was the *TaqI* - *TaqI* fragment of 1093 bp containing the 5' portion of the cDNA. The plasmid vector pUC8 (Pharmacia-LKB Biotechnology, Pharmacia House, Midsummer Boulevard, Central Milton Keynes, Bucks, MK9 3HP, UK) was digested with *AccI* and *PstI*, phenol/chloroform extracted and DNA recovered by ethanol precipitation. The 300 bp *TaqI* - *PstI* fragment from p8α1ppg was ligated using T4 DNA ligase to pUC8 cut with *AccI* and *PstI* and the ligation products were used to transform *E. coli* strain DH-1 (Gibco-BRL, PO Box 35, Trident House, Renfrew Road, Paisley PA3 4EF, Scotland, UK) to ampicillin resistance. Plasmid DNA was isolated from ampicillin resistant colonies. The correct recombinants were identified by the release of a fragment of approximately 300 bp on double digestion with *AccI* and *PstI*. The plasmid generated was called

pUC8.3'AT.3.

Plasmid pUC8.3'AT.3 was subjected to partial digestion with *Bst*NI and the fragment(s) corresponding to linearised pUC8.3'AT.3 isolated from an agarose gel. There are seven *Bst*NI sites in pUC.3'AT.3, five in the vector and two in the region corresponding to the 3'-untranslated sequences of α_1-antitrypsin. The *Bst*NI linearised and gel purified DNA was digested with *Pst*I which cuts in the pUC8 polylinker where it joins the 3' end of the cDNA insert. The *Pst*I digested DNA was end repaired with T4 DNA polymerase in the presence of excess dNTPs and self-ligated with T4 DNA ligase. The *Bst*NI - *Pst*I fragment containing the polyadenylation signal sequence is lost by this procedure. The ligated material was used to transform *E. coli* strain DH-1 to ampicillin resistance. Plasmid DNA was isolated from ampicillin resistant colonies. The correct clone was identified by restriction analysis and comparison with pUC8.3'AT.3. The correct clone was characterised by retention of single sites for *Bam*HI and *Hin*dIII, loss of a *Pst*I site, and a reduction in the size of the small *Pvu*II fragment. The correct clone was termed *pB5*.

Plasmid pB5 DNA was digested with *Acc*I, phenol/chloroform extracted and DNA recovered by ethanol precipitation. *Acc*I cleaved pB5 DNA was treated with calf intestine alkaline phosphatase (BCL). The reaction was stopped by adding EDTA to 10 millimolar and heating at 65°C for 10 minutes. The DNA was recovered after two phenol/chloroform and one chloroform extractions by precipitation with ethanol. T4 DNA ligase was used to ligate the 1093 bp *Taq*I - *Taq*I fragment described above to pB5, *Acc*I cleaved and phosphatased DNA and the ligation products were used to transform *E. coli* strain HB101 (Gibco-BRL) to ampicillin resistance. The identity of the correct clone (*pUC8∝1AT.73*) was verified by restriction analysis - presence of a 909 bp *Hin*fI fragment, a 1093 bp *Taq*I fragment, and a 87 bp *Bam*HI fragment.

Plasmid pBJ12

Plasmid pUC8α1AT.73 was digested with *Acc*I and *Hin*dIII and the resulting fragment containing the α_1-antitrypsin cDNA minus its polyadenylation signal was end-repaired using Klenow fragment of *E. coli* DNA polymerase in the presence of excess dNTPs. This blunt ended fragment was gel purified and ligated using T4 DNA ligase to gel purified pBJ8 linearised with *Pvu*II. After transformation of competent *E. coli* strain DH5α (Gibco-BRL) the correct clone was identified by restriction enzyme analysis.

Plasmid pSS1tgSpS

The gel purified *Sal*I - *Sph*I restriction fragment of approximately 4.2 kb isolated from pSS1tgXS (described in WO-A-8800239) was ligated, using T4 DNA ligase, with

equimolar amounts of gel purified pPolyI (Lathe, Vilotte & Clark, 1987, *Gene* **57**, 193-201) digested with *Sal*I and *Sph*I. [The vector pPolyI is freely available from Professor R. Lathe, LGME-CNRS and U184 INSERM, 11 rue Humann, 67085 Strasbourg, France.] After transformation of competent *E. coli* strain DH1 (Gibco-BRL) the correct clone was identified by restriction analysis.

Plasmid pBJ1

Plasmid pSS1tgSpS was digested with *Bgl*II and end-repaired using the Klenow fragment of *E. coli* DNA polymerase in the presence of excess dNTPs. The blunt-ends were modified using *Hin*dIII synthetic linkers (New England Biolabs Inc, 32 Tozer Road, Beverly, MA 01915-5510, USA) and the resulting fragment self-ligated using T4 DNA ligase in the presence of 1mM hexamine cobalt chloride, 25mM KCl (to encourage self-ligation [Rusche & Howard-Flanders (1985) *Nucleic Acids Research* **13**, 1997-2008)]. After transformation of competent *E. coli* strain DH5α (Gibco-BRL) the correct clone was identified by restriction enzyme analysis.

Plasmid pBJ16 (AATD)

The gel purified *Hin*dIII - *Sph*I fragment from pBJ1 and the gel purified *Sph*I - *Xba*I fragment from pBJ12 were ligated using T4 DNA ligase to gel purified pUC19 (Pharmacia-LKB Biotechnology, Pharmacia House, Midsummer Boulevard, Central Milton Keynes, Bucks, MK9 3HP, UK) digested with *Hin*dIII and *Xba*I. After transformation of competent *E. coli* strain DH5α (Gibco-BRL) the correct clone was identified by restriction enzyme analysis.

Isolation of AAT-D fragment from pBJ16 for microinjection

Plasmid pBJ16 was digested with *Hin*dIII and *Xba*I and the resulting 8.0 kb AATD fragment was isolated from a gel using DE81 paper (Dretzen *et al* (1981) *Analytical Biochemistry* **112**, 285-298). After separation from the DE81 paper the DNA was phenol/chloroform extracted, ethanol precipitated and finally resuspended in TE buffer (10 mM Tris-HCl, 1mM EDTA pH 8) ready for microinjection.

COMPARATIVE EXAMPLE 5

The AATD construct described in Comparative Example 4 above was used to generate transgenic mice by the method described in Example 1 of WO-A-9005188. RNA isolated from various tissues was examined for the presence of AATD transcripts and milk from the females was assayed for the presence of α_1-antitrypsin by Western blotting; both of these analyses are as described in Example 2 of WO-A-9005188. The results are shown in Table 1 below:

TABLE 1

Mice	Description	RNA	AAT Protein*
AATD12	G0 female	-	-
AATD14	G0 female	-	-
AATD31	G0 female	-	-
AATD33	G0 female	-	3.9 μg/ml
AATD9	mouse-line	-	-
AAT21	mouse-line	-	-
AATD41	mouse-line	-	-
AATD47	mouse-line	-	-

*assessed by Western blotting and/or ELISA

Only 1/8 of the transgenic mice carrying AATD expressed the transgene as determined by analysis of milk proteins. The level of expression was quite low and despite repeated attempts no corresponding AATD RNA transcripts were detected in the mammary gland.

EXAMPLE 1

The β-lactoglobulin construct prepared in Comparative Examples 1 and 3, containing the 10.5 kb *SalI-XbaI* fragment prepared from pSS1tgXS ("BLG") and the α_1-antitrypsin construct prepared in Comparative Example 3 ("AATD") were co-injected into mouse eggs as before in an equimolar ratio in a total DNA concentration of about 3 μg/ml. 20 transgenic founder mice were detected by Southern blotting and 11 of these were found to carry both AATD and BLG sequences. These mice were designated BAD (BLG and AATD). 9/11 transmitted both transgenes to the G1 progeny. Analysing a number of progeny in each line showed that in each case the two transgenes had segregated together (Figure 5) indicating that they were integrated very close together and in all probability were co-integrated at the same site.

Figure 5 shows the co-segregation of AATD and BLG. DNA samples from the various BAD lines resulting from co-injection with AATD and BLG were restricted with *Eco*RI, Southern blotted and probed with an *Eco*RI-*Sph*I fragment that hybridised to the 5' end of the two transgenes. BLG and AATD transgenes

can be distinguished by *Eco*RI digestion and the specific *Eco*RI fragments are indicated. DNA from a number of G1 animals has been analysed. The similar pattern of AATD and BLG restriction fragments in G1 animals from the same line (ie BAD 1; BAD99 etc) is indicative of co-segregation. Note that line BAD93 may have two co-integrated loci which are segregating in the G1 generation.

Expression of AATD mRNA has been analysed in all of the 9 lines of the double transgenic mice. AATD transcripts of the expected size were detected in mammary RNA in 6 out of the 9 lines: 3 lines expressed AATD mRNA at low levels, 1 at medium level and 2 at high level. AATD transcripts were not detected in other tissues (Figure 6).

Figure 6 shows tissue-specific expression of AATD in BAD lines. RNA prepared from a variety of tissues was probed with human AAT-specific sequences. The ~1600 nt AATD transcripts (arrowed D) are seen specifically in the mammary gland of BAD mice 99.3 and BAD 135.13. The ~1400 nt transcripts from transgene AATB (arrowed B) expressed in the mammary gland) in transgenic line AATB 35 (35M); CM, control mouse mammary gland RNA (see also Example 2 of WO-A-9005188). Sa; salivary gland; H, heart; K, kidney; Sp, spleen; L, liver; M, mammary gland.

Human α_1-antitrypsin has been measured in the two high expressing lines and estimated at about 140 μg/ml and about 600 μg/ml (mean values of two) respectively. The BLG transgene has also been analysed for expression: the 6 lines that expressed AATD mRNA also expressed BLG mRNA, whereas the three AATD lines apparently negative for AATD mRNA do not apparently express BLG (representative examples shown in Figure 7). Figure 7 illustrates detection of AATD and BLG transcripts in BAD mice. Mammary gland (M) and liver (L) RNA samples, blotted onto Hybond membranes, were probed with AAT (α1AT).

~1600 nt AATD transcripts are detectable in mammary gland RNA samples from BAD mice 1.1, 1.2, 36.8, 107.2, 158.3, 99, 99.3 and 135.13. The filters were stripped and re-hybridised to a BLG-specific probe (BLG); the same samples showed strong hybridisation to the ~800 nt BLG transcript, also detected in sheep mammary gland RNA (SM); AATB 35, mammary gland RNA from transgenic line AATB 35 containing ~1400 nt AATB transcript; CM, control mouse samples.

It therefore appears that at least for the most part AATD expression may be associated with an actively expressing BLG gene. Comparing the results of this example with those of Comparative Example 5, it appears that the co-injection (and presumably co-integration) of AATD with BLG has significantly increased the efficiency of expression of AATD. Considering RNA data only, when AATD was injected alone 0/8 animals expressed the transgene. When co-injected (and presumable co-integrated) with BLG, expression of AATD mRNA was detected in 6/9 animals as shown in Table 2 below.

TABLE 2

Mouse/ Line	RNA[1] αlAT	RNA[1] BLG	Protein αlAT[2] (μg/ml)	Protein BLG[3] (mg/ml)
BAD 1	+	+	4.8	9
BAD 36	+	+	1.2	1.1
BAD 41	-	-	-	-
BAD 93	-	-	(.84)[4]	-
BAD 99	+	+	137	1.7
BAD 107	+	+	24	2.4
BAD 135	+	+	610	1.7
BAD 144	-	-	-	-
BAD 158	+	+	1	1.6

Expression in BAD Transgenic Mice

1. mRNA transcripts detected on N. blots of total and poly A+ RNA.
2. αlAT determined by ELISA: sensitivity >0.2 μg/ml. For BAD lines

values represent average from 2 mice.

3. BLG estimated densitometrically on SDS-PAGE gels by reference to BLG standard.

4. Expression detected in only one out of two animals from this line.

COMPARATIVE EXAMPLE 6

The procedure of Example 4 (construction of AATD) is repeated, except that the DNA sequence encoding the polypeptide of interest encodes Factor IX. A *Nhe*I-*Hin*dIII fragment comprising 1553 bp of the insert from p5'G3'CV1 [see WO-A-8800239] was inserted into the *Pvu*II site of pBJ8 as described above for pBJ12 to generate FIX D.

COMPARATIVE EXAMPLE 7

The FIX construct described in Comparative Example 6 above was used to generate transgenic mice by the method described in Example 1 of WO-A-9005188. RNA isolated from various tissues was examined for the presence of FIX transcripts and milk from the females was assayed for the presence of factor IX by Western blotting and/or ELISA; both of these analyses are as described in Example 2 of WO-A-9005188. The results are shown in Table 3 below:

TABLE 3

Mice	Description	RNA	FIX Protein*
FIX D 11	GO female	-	-
FIX D 14	GO female	-	-
FIX D 16	GO female	-	-
FIX D 17	GO female	-	-
FIX D 18	GO female	-	-
FIX D 20	mouse-line	-	-
FIX D 23	mouse-line	-	-
FIX D 24	mouse-line	-	-
FIX D 26	mouse-line	-	-

*assessed by Western blotting and/or ELISA

None of the transgenic mice carrying FIX D expressed the transgene as determined by analysis of milk proteins or by Northern blotting of mammary gland RNA.

EXAMPLE 2

The β-lactoglobulin construct prepared in Comparative Examples 1 and 3, containing the 10.5kb *SalI-XbaI* fragment prepared from pSS1tgXS ("BLG") and the factor IX construct prepared in Comparative Example 3 ("FIX D"), were co-injected into mouse eggs as before but in a molar ratio of 3 BLG : 1 FIX D in a total DNA concentration of about 3μg/ml. 30 transgenic founder mice were detected by Southern blotting and 20 of these were found to carry both FIX and BLG sequences. These mice were designated BIX (BLG and FIX D). 12/13 transmitted both transgenes to the G1 progeny. Analysing a number of progeny in each line showed that in each case the two transgenes had segregated together (Figure 8) indicating that they were integrated very close together and in all probability were co-integrated at the same site.

Figure 8 shows the co-segregation of FIX D and BLG. DNA samples from the various BIX lines resulting from co-injection with FIX D and BLG were restricted with *Bam*HI, Southern blotted and probed with an *Eco*RI-*Sph*I fragment that hybridised to the 5' end of the two transgenes. BLG and FIX D transgenes can be distinguished by *Bam*HI digestion and the specific *Bam*HI fragments are indicated. DNA from a number of G1 animals has been analysed. The similar pattern of FIX D and BLG restriction fragments in G1 animals from the same line (eg BIX 34; BIX 99 etc) is indicative of co-segregation. Note that line BIX 29 may have two co-integrated loci which are segregating in the G1 generation.

Expression of FIXD mRNA has been analysed in 11 of the 12 lines of the double transgenic mice. The FIX D transcripts of the expected size were detected in

mammary RNA in all these lines: 4 lines expressed FIX mRNA at low levels, 4 at medium level and 3 at high level. (Some variation in the level of expression between individual mice of a given line was observed in some cases.) FIX D transcripts were not detected in other tissues (Figure 9).

Figure 9 shows tissue-specific expression of FIX D in BIX lines. RNA prepared from a variety of tissues was probed with fIX-specific sequences. The ~1800 nt FIX D transcripts (arrowed) are seen specifically in the mammary gland of BIX mice 12.1, 30.2 and 33.1. The ~2600 nt transcript from the endogenous FIX gene (arrowed B) is present in liver samples, CM, control mouse mammary gland RNA (see also Example 2 of WO-A-9005188) CL, control liver RNA. Sa; salivary gland; H, heart; K, kidney; Sp, spleen; L, liver; M, mammary gland. FIX A51, mammary gland RNA from a transgenic mouse carrying a second FIX transgene, FIX A that expresses a ~2400nt FIX transcript (arrowed A). Human factor IX was detected in the milk from mice from 6 out of the 10 lines which have been analysed - see Table 4 below. The BLG transgene has also been analysed for expression; all the lines analysed were shown to express BLG mRNA also expressed BLG mRNA.

Figure 10 illustrates detection of FIX D and BLG transcripts in BIX mice. Mammary gland (M) and liver (L) RNA samples, blotted onto Hybond membranes, were probed with fIX specific sequences. ~1800 nt FIX D transcripts are detectable in mammary gland RNA samples from BIX mice 34.1, 37.1, 43.3, 66.2, 10.3, 12.1, 30.4, 33.1, 22.13, 29 and 131.2. The filters sere stripped and re-hybridised to a BLG-specific probe (BLG); the same samples showed hybridisation to the ~800 nt BLG transcript, FIX A 51, mammary gland RNA from transgenic mouse FIX A 51 containing ~2400 nt FIX A transcript; C, control mouse samples.

Comparing the results of this example with those of Comparative Example 6, it appears that the co-injection (and presumably co-integration) of FIX D with BLG has significantly increased the efficiency of expression of FIX D. Considering RNA data only, when FIX D was injected alone, 0/9 mice lines expressed the transgene. When injected (and presumably co-integrated) with BLG, expression of FIX D mRNA was detected in 11/11 lines as shown in Table 4 and FIX proteins detected in the milk of a further line for which RNA was not available.

TABLE 4

Expression in BIX transgenic mice

BIX				
Mouse/ Line	RNA[1]		Protein	
	fIX	BLG	fIX[2]	BLG[3]
10	+	+	-	+
12	+	+	+	+
22	+	+	-	+
29	+	+	+	+
30	+	+	+	+
32	n.d.	n.d.	+	+
33	+	+	+	+
34	+	+	-	+
37	+	+	n.d.	+
43	+	+	n.d.	+
66	+	+	-	+
131	+	+	+	+

Notes:

1. mRNA transcripts detected on N blots of total mammary gland RNA
2. fIX detected by W blotting and/or ELISA
3. BLG detected on Coomassie blue stained SDS-PAGE gels.

n.d. not done.

EXAMPLE 3

A β-lactoglobulin/bovine α-lactalbumin construct (BLG/alacTR.D -- BAT) was prepared in the following way. The 3.0kb *Xho*I fragment comprising regulatory sequences derived from the bovine α-lactalbumin gene fused to a segment of DNA encoding ovine trophoblastin was excised from the plasmid vector (for full details of this plasmid construct see Stinnakre *et al* (1991), FEBS Letters, 284 1, 19-22). This fragment was inserted directly into the plasmid pSS1tgXS (see WO-A-8800239) at the unique *Sal*I site (Figure 11). The resulting 13.5kb insert comprising the alacTR gene linked to the BLG gene was excised from the plasmid vector by digestion with *Xba*I. This fragment was purified by gel electrophoresis and used for the direct microinjection of mouse eggs, as previously described.

EXAMPLE 4

All references to the DNA sequence of the β-lactoglobulin gene utilise the numbering of the sequence allocated EMBL Accession No. XI2817 (Harris *et al*, *NAR* 16:10379-80 (1988)).

Plasmid pUC.PM

The multiple cloning site of the vector pUC18 (Yanisch-Perron *et al*, (1985) *Gene* 33:103-119) was removed and replaced with a synthetic, double stranded, oligonucleotide containing the new restriction sites: *Pvu*I/*M1u*I/*Sal*I/*Eco*RV/*Xba*I/*Pvu*I/*M1u*I, and flanked by 5'-overhangs compatible with the restriction sites *Eco*RI and *Hin*dIII. pUC18 DNA was cleaved with both *Eco*RI and *Hin*dIII, the 5'-terminal phosphate groups were removed with Calf Intestinal Phosphatase to prevent religation of this starting material. The new linker DNA was ligated into pUC18. The DNA sequence across the new multiple cloning site was confirmed. This new vector was called pUC.PM.

Plasmid pUCXS

The β-lactoglobulin gene sequences from the plasmid pSS1tgXS (see Comparative Example 3 above) were excised on a *SalI* and *XbaI* fragment and recloned into the vector pUC.PM, cut with *SalI* and *XbaI*, to give plasmid pUCXS.

Plasmid pUCXS/RV

The plasmid pSS1tgSE (see WO-A-8800239) contains β-lactoglobulin gene sequences from the *SphI* site at position 754 to the *Eco*RI site at 2050, a region spanning a unique *NotI* site at position 1148. This insert contains a single *PvuII* site (832) which lies in the 5'-untranslated of the β-lactoglobulin mRNA. Into this site was blunt-end ligated a double stranded, 8bp, DNA linker encoding the recognition site for the enzyme *Eco*RV, to give the plasmid pSS1tgSE/RV. The DNA sequences bounded by *SphI* and *NotI* were then excised and used to replace the equivalent fragment in the plasmid pUCXS, thus effectively introducing a unique *Eco*RV site into the β-lactoglobulin gene placed in such a way as to allow the insertion of any additional DNA sequences under the control of the β-lactoglobulin gene promoter and 3' to the initiation of transcription. The resulting plasmid was called pUCXS/RV.

Plasmid pUCSV

A derivative of pUCXS/RV, containing only the 4.2kbp of the β-lactoglobulin gene which lie 5' to the transcription initiation site (the promoter), was constructed by subcloning the *SalI-Eco*RV fragment into pUC.PM; this plasmid is called pUCSV.

Plasmid pBLAC100

A fragment of the 3' flanking sequence of the β-lactoglobulin gene were subcloned in such a way as to eliminate all introns. Plasmid DNA of pUCXS/RV which was partially digested with *SmaI* by performing an enzyme titration with lower and lower concentrations of enzyme at a fixed concentration of DNA. The *SmaI*

protein was removed by phenol-chloroform extraction and ethanol precipitation and the DNA resuspended in water. This DNA was subsequently digested to completion with the enzyme *Xba*I. DNA cut once at the *Sma*I site, position 5286, and then cleaved with *Xba*I gave a characteristic band of size 2.1kbp. This band was purified from an agarose gel slice and ligated into *Sma*I and *Xba*I cut pBSIISK+ (Stratagene Ltd, Cambridge Science Park, Cambridge, UK) to give the plasmid pBLAC100.

Plasmid pMAD

The β-lactoglobulin cloning vector pMAD was constructed to allow rapid insertion of cDNAs under the control of the β-lactoglobulin gene promoter and 3'-flanking sequences. Such constructs contain no introns. The plasmid pBLAC100 was opened by digestion with both *Eco*RV and *Sal*I, the vector fragment was gel purified. Into this was ligated the 4.2kbp promoter fragment from the plasmid pUCSV as a *Sal*I-*Eco*RV fragment. This construct is termed pST1 and constitutes a β-lactoglobulin mini-gene encoding the 4.2kbp promoter and 21kbp of 3'-flanking sequences. A unique *Eco*RV site is present to allow blunt-end cloning of any additional DNA sequences. In order to allow excision of novel β-lactoglobulin gene constructs with the enzyme *M1u*I the entire mini-gene from pST1 was excised on a *Xho*I-*Not*I fragment, the DNA termini made flush with Klenow polymerase, under standard conditions, and blunt-end cloned into the *Eco*RV site of pUC.PM to give pMAD.

Plasmid pCORP2

A 1450bp cDNA of the protein C gene, flanked by *Kpn*I sites, was obtained in the form of plasmid pWAPC2. The cDNA was excised as a *Kpn*I fragment, the 3' overhangs made flush by treatment with T4 DNA polymerase, the fragment gel purified and blunt-end cloned into the *Eco*RV site of pMAD. Orientation was determined by restriction digest and confirmed by DNA sequencing. This

construct is plasmid pCORP2 and contains the protein C cDNA under the transcriptional control of the β-lactoglobulin gene 5' and 3' flanking sequences. There are no introns.

Isolation of CORP2 fragment from pCORP2 for microinjection

Plasmid pCORP2 was digested to completion with *MluI* and the resulting 7.75kbp CORP2 fragment was excised from a 1% agarose gel after electrophoretic separation. The DNA was purified from the agarose slice using the PREPAGENE® kit (Biorad) and eluted in TE buffer ready for dilution and microinjection.

Transgenic Mouse Production

Transgenic mice were generated by direct pro-nuclear injection essentially as described by Gordon and Ruddle, "Methods in Enzymology", Vol 101, [(1983) Eds. Wu, Grossman and Moldave], Academic Press pp 411-432. The 'β-Lactoglobulin' construct and the 'Protein C' construct were co-injected at both equimolar ratio and non-equimolar ratio (1:3 with respect to Protein C) at an overall concentration of 6μg/ml. 12 founder mice were produced, all of which had integrated both transgenes.

Injection of CORP2 DNA alone into mouse eggs resulted in the generation of 11 lines of transgenic mice. Milk was collected from these mice and analysed for the presence of Protein C by ELISA. The ELISA was based on the double antibody sandwich principle and used rabbit polyclonal antibodies raised against human plasma derived Protein C. The ELISA is capable of measuring Protein C levels as low as 100ng/ml but was unable to detect Protein C in any of the 11 lines of transgenic CORP2 mouse milks (see Table 5).

TABLE 5

Levels of human protein C in the milk of transgenic mice containing th CORP 2 construction alone.

Mouse Line	Protein C Concentration
94-15	-ve
94-13	-ve
94-4	-ve
94-9	-ve
95-3	-ve
97-4	-ve
96-28	-ve
95-10	-ve
96-26	-ve
97-15	-ve
97-22	-ve

Co-injection of the CORP 2 construct with the ovine β-lactoglobulin gene (UCXS/RV) resulted in the generation of three transgenic lines. Table 6 shows the levels of Protein C in milk samples taken from these mice as measured by the ELISA described above.

TABLE 6

Levels of human Protein C in the milk of transgenic mice containing both the CORP 2 construct and ovine β-lactoglobulin gene (UCXS/RV).

Mouse Line	Protein C Concentration
99-2	1.7μg/ml
102-7	15μg/ml
103-4	- ve

CLAIMS

1. A process for the preparation of a transgenic animal or plant capable of expressing a first DNA sequence, the process comprising co-introducing into a cell or group of cells from which an animal or plant may be derived the first DNA sequence and a second DNA sequence, wherein the second DNA sequence is, when so introduced without the first sequence, capable of being expressed as, or regulating the expression of, a transgene with greater specificity and/or with a greater frequency of expression and/or at a higher level than that at which the first sequence, without the second sequence, is capable of being expressed as a transgene, and allowing a transgenic animal to develop from the cell(s).

2. A process as claimed in claim 1, wherein the method of introducing DNA into an animal cell is injection.

3. A process as claimed in claim 1 or 2, wherein the first and second DNA sequences are co-introduced by introducing a mixture of them into the recipient cell.

4. A process as claimed in claim 1 or 2, wherein co-introduction is achieved by introducing covalently or otherwise linked first and second DNA sequences into the recipient cell.

5. A process as claimed in any one of claims 1 to 4, wherein the first DNA sequence comprises cDNA or other DNA encoding a desired protein and sufficient regulatory sequences operatively linked to the protein-encoding DNA to direct the expression.

6. A process as claimed in claim 5, wherein the protein has pharmaceutical activity.

7. A process as claimed in any one of claims 1 to 6, wherein the second sequence is derived from a gene, normally expressed in a target organ or tissue.

8. A process as claimed in claim 7, wherein the target organ for expression is the mammary gland of a transgenic mammal and the second DNA sequence is derived from a milk protein gene.

9. A process according to claim 8, wherein the gene from which the second DNA sequence is derived is the same as that from which regulatory sequences used in the first DNA sequence are derived.

10. DNA useful for the preparation of a transgenic animal or plant expressing a first DNA sequence, the DNA comprising, on the same or separate molecules, the first sequence and a second DNA sequence, wherein the second DNA sequence is, when introduced as a transgene without the first sequence, capable of being expressed, or capable of regulating expression, with greater specificity and/or a greater frequency of expression and/or at a higher level than that at which the first sequence, when introduced as a transgene without the second sequence, is capable of being expressed.

11. A transgenic animal or plant capable of expressing a first sequence as a transgene, the said animal or plant having the first sequence and a second sequence, wherein the second sequence is such that, when introduced as a transgene without the first sequence, it is capable of being expressed or regulating expression with greater specificity and/or with a greater frequency of expression and/or at a higher level than that at which the first sequence, when introduced as a transgene without the second sequence, is capable of being expressed.

FIG. 1

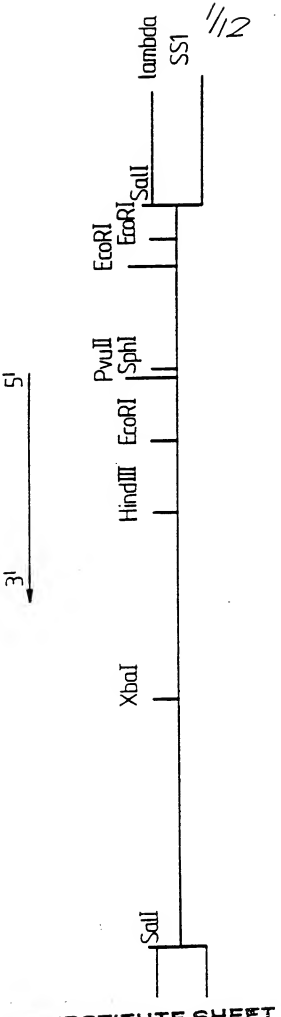

lambda
SS1
SalI
EcoRI
EcoRI
PvuII
SphI
EcoRI
HindIII
XbaI
SalI
5'
3'
1kb

FIG. 2

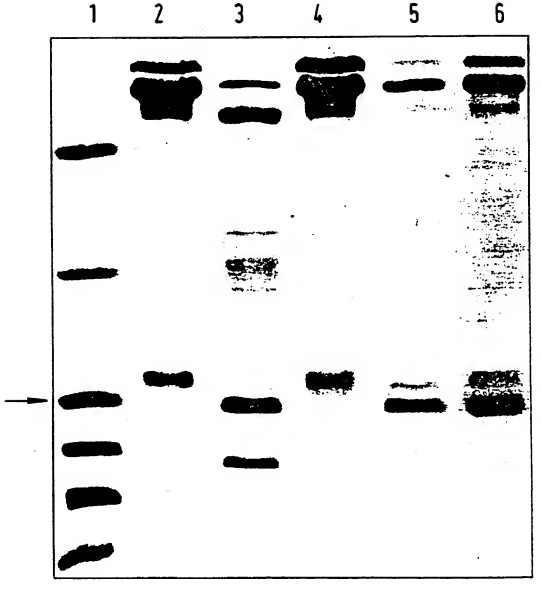


FIG. 3

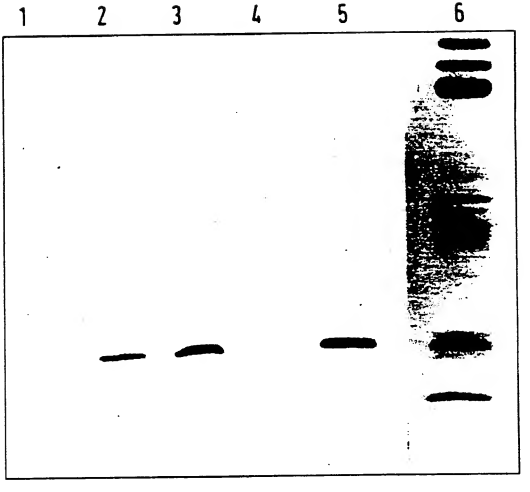


FIG. 4a

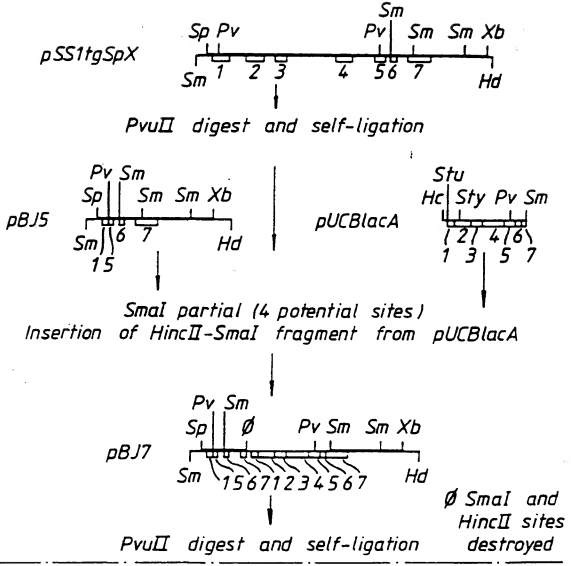
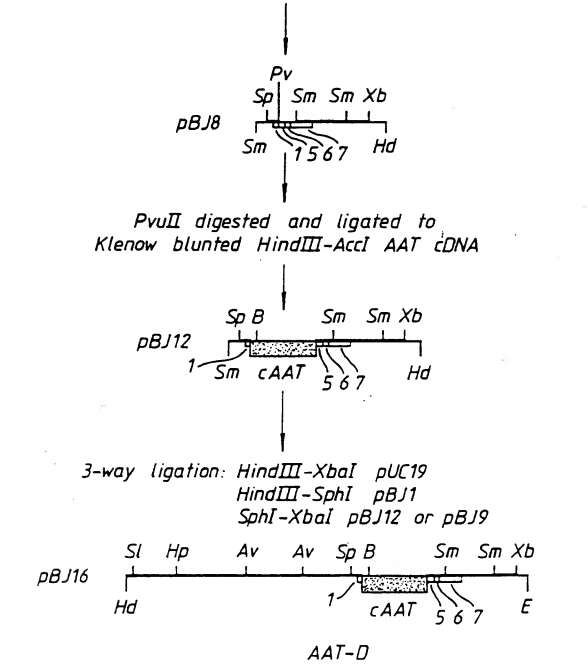
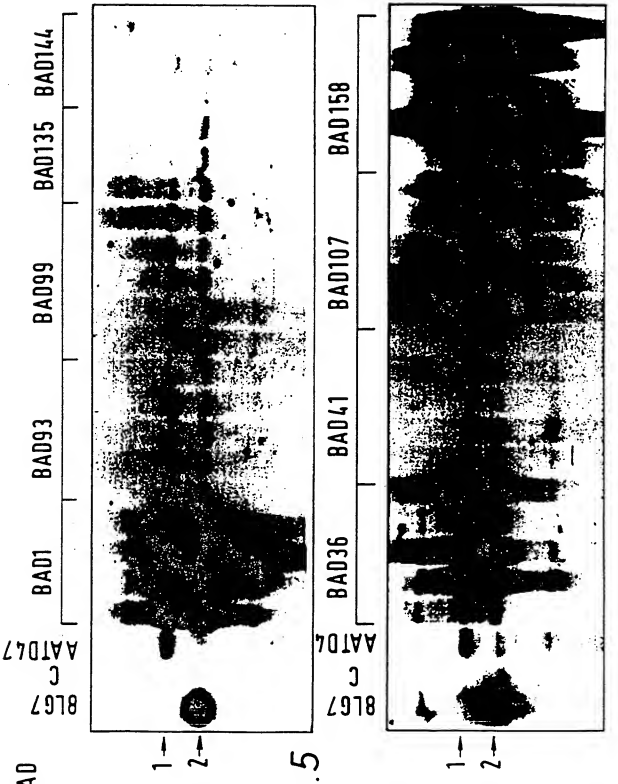

pSS1tgSpX
Sp Pv
Sm
Pv Sm Sm Xb
Sm 1 2 3 4 5 6 7 Hd
PvuII digest and self-ligation
pBJ5
Pv Sm
Sp Sm Sm Xb
Sm 6 7 Hd
1 5
pUCBlacA
Stu
Hc Sty Pv Sm
1 2 3 4 5 6 7
SmaI partial (4 potential sites)
Insertion of HincII-SmaI fragment from pUCBlacA
pBJ7
Pv Sm
Sp Ø Pv Sm Sm Xb
Sm 1 5 6 7 1 2 3 4 5 6 7 Hd
PvuII digest and self-ligation
Ø SmaI and HincII sites destroyed

FIG. 4b


Pv
Sp
Sm Sm Xb
pBJ8
Sm
1 5 6 7
Hd
PvuII digested and ligated to
Klenow blunted HindIII-AccI AAT cDNA
Sp B
Sm Sm Xb
pBJ12
1
Sm cAAT
5 6 7
Hd
3-way ligation: HindIII-XbaI pUC19
HindIII-SphI pBJ1
SphI-XbaI pBJ12 or pBJ9
Sl Hp Av Av Sp B Sm Sm Xb
pBJ16
Hd
1
cAAT
5 6 7
E
AAT-D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BLG7
C
AATDL7
BAD1
BAD93
BAD99
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BAD144
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2
BLG7
C
AATDL
BAD36
BAD41
BAD107
BAD158
1
2

FIG.5

FIG. 6

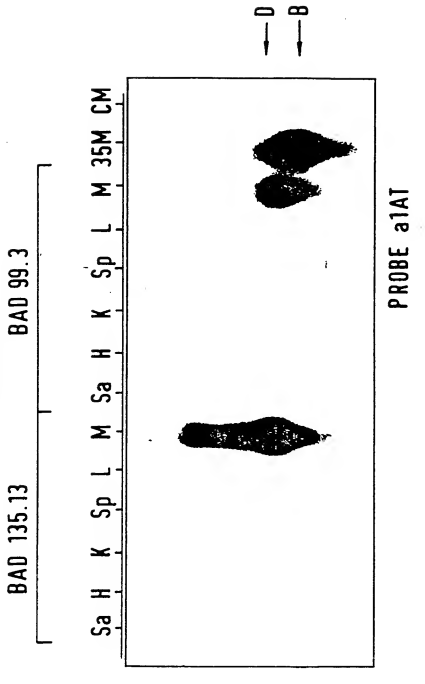


FIG. 7

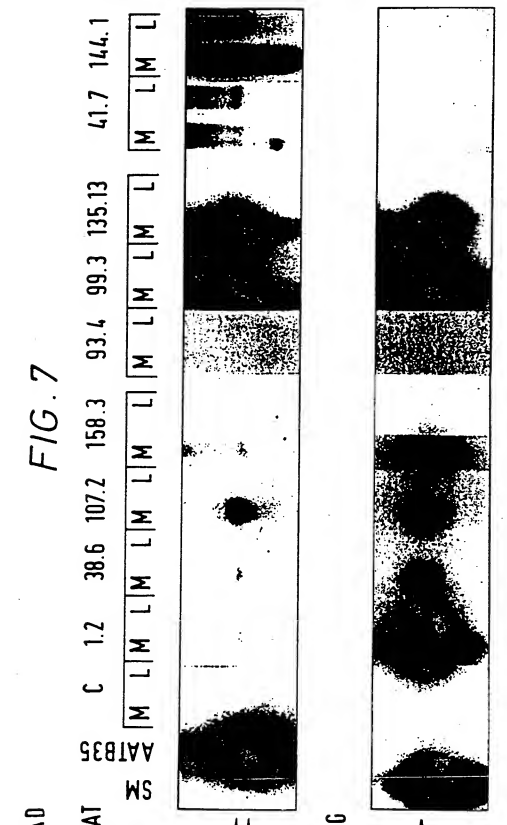

BAD
α1AT
SM
AATB35
C 1.2 38.6 107.2 158.3 93.4 99.3 135.13 41.7 144.1
M L M L M L M L M L M L M L M L M L
1
2
BLG
3

FIG. 8

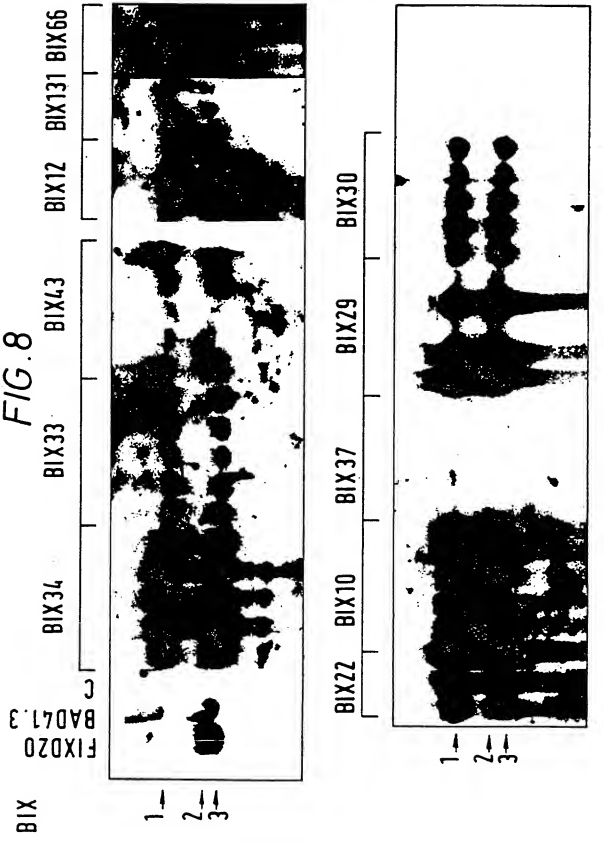

BIX
FIX020
BAD41.3
C
BIX34
BIX33
BIX43
BIX12
BIX131
BIX66
1
2
3
BIX22
BIX10
BIX37
BIX29
BIX30
1
2
3

FIG. 9

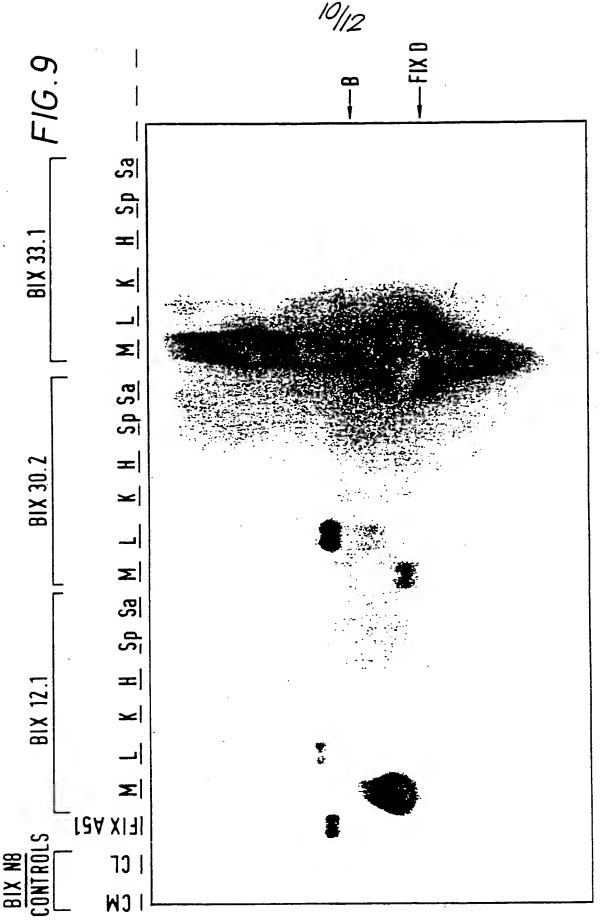
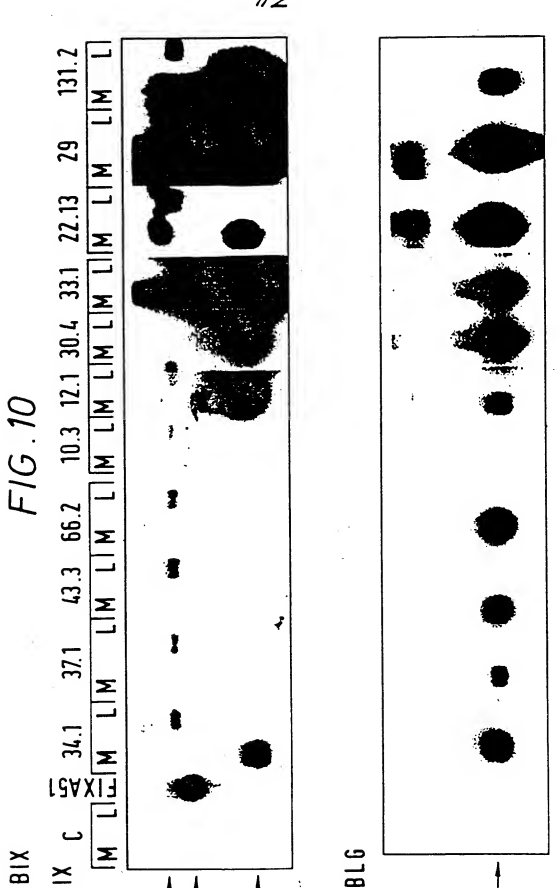
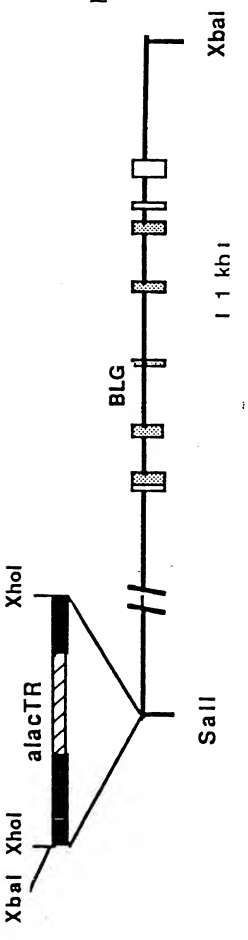

BIX NB CONTROLS
CM
CL
IFIX A51
M L K H Sp Sa M L K H Sp Sa M L K H Sp Sa
BIX 12.1
BIX 30.2
BIX 33.1
B
FIX D
PROBE:p5'G3'CVI

FIG. 10


BIX
fIX
C
XA51
F
M
L
34.1
37.1
43.3
66.2
10.3
12.1
30.4
33.1
22.13
29
131.2
1
2
3
BLG
4

12/12

FIG. 11



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/02318

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/00; C12N15/85; C12N15/82; A01K67/00
C12N9/64; C12N15/12; C12N15/15; C12N15/89

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	WO,A,8 800 239 (PHARMACEUTICAL PROTEINS LTD) 14 January 1988 cited in the application see page 19, line 1 - page 21, line 1; claims	1,2,4-11
X	WO,A,9 005 188 (PHARMACEUTICAL PROTEINS LTD) 17 May 1990 cited in the application see page 6, line 1 - page 7, line 24; claims; figures 1-10,16-20; example 1	1,2,4-11
P,X	EP,A,0 420 055 (W. R. GRACE & CO.-CONN.) 3 April 1991 see the whole document	1,2,4-11
X	WO,A,9 007 936 (CHIRON CORPORATION) 26 July 1990 see the whole document	1,2,4,5,7,10

-/--

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
01 APRIL 1992	09 APR 1992
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CHAMBONNET F.J.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

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X	WO,A,9 013 645 (SOMATOGENETICS INTERNATIONAL CORP.) 15 November 1990 see the whole document	1,3,4-6, 9,10
P,X	WO,A,9 115 111 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 17 October 1991 see the whole document	1,2,4,5, 10,11
P,X	WO,A,9 106 666 (CELL GENESYS, INC) 16 May 1991 see the whole document	1,4-6,10
P,X	WO,A,9 113 151 (BIOGEN, INC.) 5 September 1991 see the whole document	1,2,4, 6-8,10, 11
P,X	EXPERIENTIA vol. 47, no. 9, 15 September 1991, BASEL, CH pages 905 - 912; WILMUT, J. ET AL.: 'Production of pharmaceutical proteins in milk' Abstract, lines 10-11 see page 908, column 1, line 22 - page 912	12,4-11

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. GB 9102318
SA 55103

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The members are as contained in the European Patent Office EDP file on
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		WO-A-	9106667	16-05-91
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